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RESEARCH ARTICLE

Androgen Derivates Affect Immunopresence but not Activity of Antioxidant Enzymes in Pancreatic Islets during Diabetogen-Induced Apoptosis

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Abstract:

The onset of diabetes mellitus involves oxidative stress. Previous reports from our laboratory have shown that androgens, but not estrogens or progestogens, could prevent the apparition of apoptosis in beta-cell induced by streptozotocin in early stages of diabetes induction in rat; these effect was related with changes in antioxidant enzymes presence. The aim of this study was to evaluate the participation of scavenging enzymes in beta-cells protection under androgen derivatives, and their cytoprotective mechanism. Adult male rats were orchidectomyzed, and treated with androgens after STZ administration. Pancreatic catalase and superoxide dismutase-1 and -2 were analyzed by immunohistochemistry and TUNEL assay. In another experimental focusing, pancreatic islets were obtained from intact male rats, and incubated with alloxan in order to induce ROS production, in the presence of androgen derivatives, and the activities of catalase, superoxide dismutase and glutathione peroxidase were measured in cell lysates. Androgen treatment affects immunopresence of the antioxidant enzymes in pancreas, but not their activity in islets, suggesting that scavenging enzymes could be affected during beta-cells apoptosis.

Keywords: Pancreatic islets, scavenging enzymes, androgen derivatives, apoptosis, immunohistochemistry

Introduction

Diabetes mellitus (DM) is the clinical expression of absolute or relative insulin deficiency, characterized by carbohydrate, lipid, and protein metabolism alterations. Type-1 diabetes mellitus (DM1) is characterized by hyperglycemia-hyperketonemia, diminished insulin production, and a general disturbance of metabolism; meanwhile type-2 DM is developed after islet dysfunction, glucose intolerance and tissue resistance to insulin (Diabetes Control and Complications Trial Research Group, 1993; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

Antioxidant or scavenging enzymes reduce the levels of reactive oxygen species (ROS) in the cells. The first-line antioxidant enzymes are superoxide dismutase (SOD), catalase (Cat) and glutathione-peroxidase (GPx). SOD catalyzes the conversion of superoxide anion to hydrogen peroxide, which is subsequently converted to water by catalase and glutathione-peroxidase activity. The actions of these enzymes reduce oxidative stress (Poswig et al., 1999). It is well know that beta-cells are high susceptible to oxidative changes since the expression of these enzymes are very low (Hotta et al., 1998; Kajimoto and Kaneto, 2004). In the pathogenesis of DM, it has been demonstrated the role of oxidative stress. Overexpression of antioxidant enzymes targeted at β-cells protects it against ROS effects: Cu/Zn SOD activity decreases alloxan-induced diabetes (Kubisch et al., 1997), and Cat overexpression delays the development of hyperglycemia induced after one single high dose of streptozotocin (STZ) (Xu and Badr, 1999).

Steroid hormones can affect cellular susceptibility to different harmful situations like oxidative stress (Ahlbom et al., 2001). In different cells and tissues, it has been demonstrated that steroid hormones have antioxidant properties (Aragno et al., 1999; Ahlbom et al., 2001; Deroo et al, 2004; Miyake et al., 2004). Testosterone inhibits oxidative stress-induced cell death in cerebellar granule cells from (Deroo et al., 2004), and participates in defensive mechanisms of oxidative stress by increasing the levels of prostatic antioxidant enzymes in rats (Pang et al., 2002; Tam et al., 2003). The exogenous administration of dehydroepiandrosterone (DHEA) to male rat protects various organs against lipid peroxidation (Bocuzzi et al., 1997), and prevents oxidative damage caused by hyperglycemia (Aragno et al., 1999).

In animal models with DM, it has been demonstrated a sexual dimorphism associated to steroid hormones, which confers different susceptibility to damage in the pancreatic beta-cells (Paik et al., 1982; Baxter et al., 1989; Rosmalen et al., 2001; LeMay et al., 2006). Previously, our group reported that testosterone, but not progesterone or estradiol, protects pancreatic islets from STZ-induced apoptosis in male rats; and this effect was sex specific, since males but nor females respond to treatment. In the same study it was observed an inverse correlation between the apoptosis and immunopresence of Cu/Zn-SOD and Cat (Palomar-Morales et al., 2010).

For these reasons, this work was designed to study the possible effect of two androgenic derivatives, DHEA and dyhidrotestosterone (DHT) over immunopresence and activity of scavenging enzymes in pancreatic islets from rat, and to compare with the effect of the principal androgen.

Material and methods

Experimental subjects

Maintenance and handling of experimental subjects was performed in accordance with the Mexican legal guidelines of animal protection (NOM-062-ZOO-1999). Male rats were provided and stored in the bioterium of the Facultad de Estudios Superiores Iztacala until the moment of the experiment. The bioterium committee approved experimental procediments in animals.

Effect of androgen analogs over apoptosis and enzyme immunohistochemistry

Four groups of three male Wistar rats (200-250 g) were gonadectomized under ketamine (50 mg/Kg) and xylazine (5 mg/kg) anesthesia as previously described, and 72 hours later treated with the respective hormone (Sigma Chemical Co, St Louis, MO), at dose of 5 mg/Kg body weight (BW): testosterone enanthate (TE), DHT, DHEA or corn oil (Sigma Chemical Co, St Louis, MO) as vehicle; diabetes model was induced with STZ (Sigma Chemical Co, St Louis, MO) at dose of 60 mg/Kg BW in citrate buffer (100 mM, pH 4.5), as described previously (Palomar-Morales et al., 2010). At six hours after STZ treatment, rats were killed by pentobarbital overdose (60 mg/Kg body weight), and the pancreases were obtained. After rinsed, pancreases were fixed, dehydrated, cleared and embedded in Paraplast Plus (Leica Biosystems Richmond Inc, Richmond, IL), and sectioned at 8 µm in a rotation microtome (Leica RM2125 RTS, Leica Biosystems Nusslooch, Heidelberg, Deutschland), and TUNEL as well as immunohistochemistry for catalase or SOD1 (Cu/Zn-SOD) were realized as previously described (Palomar-Morales et al., 2010). Additionally, immunohistochemistry analysis for SOD2 (Mn-SOD) was realized, with the use of polyclonal rabbit anti sheep Mn-SOD as primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibody (Sinha et al., 2007). Micrographs were taken in a microscope Leica DM500 with a digital camera Leica EC3 (Leica RM2125 RTS, Leica Biosystems Nusslooch, Heidelberg, Deutschland).

Effect of androgen analogs over scavenging enzyme activity in isolated islets

Also, four groups of three male Wistar rats (200-250 g), were gonadectomized, treated with the respective hormone, and induced with STZ as described above. After sacrifice, pancreas was digested with Hanks solution supplemented with 2 mg of collagenase IV (Sigma Chemical Co, St. Louis MO), by shaking incubation at 37o C for 10 min. Under sterile conditions, in a Class II laminar flow biological safety cabinet (model BHC-12-B2, Veco, Mexico) islets were obtained in a discontinued gradient of Histopaque 1077 (Sigma Chemical Co, St. Louis MO), and then purified by resuspension cycles in a centrifuge 5804 - A-4-44 (Eppendorf, USA). The islet-rich suspension was incubated 24 h at 37º C in 5 % CO2

atmosphere in supplemented α-MEM medium, in a CO2 incubator (Model AAC-160T, Luzeren; USA).

Islets (ca. 200 per mL) were incubated in phosphate-buffered saline with 6 mM alloxan (Sigma Chemical Co, St. Louis MO), as ROS generator (Devi and Das, 2004) in the same conditions, during 6 h. At 1-h time intervals, 100 µL from supernatant media was obtained, and cells were disrupted by freeze/thawing, in order to determinate the enzymatic activities of Cat, SOD and GPx. Briefly, Cat was measured by the decrease of absorbance at 240 nm by addition of a solution of hydrogen peroxide to the crude extract (Aebi, 1983). SOD activity was quantified by the inhibition of electron transference from xanthine to nitroblue tetrazolium, catalyzed by xanthine oxidase, in alkaline pH; and the activity of SOD is expressed with regard the inhibition of NBT reduction. The xanthine oxidase and their inhibition for SOD were followed at 550 nm (Beauchamp and Fridovich, 1971). The assay of GPx is based on the reaction of reduced glutathione with hydrogen peroxide, to produce water and oxidized glutathione, that reacts with NADPH by reaction of glutathione reductase, and reduced glutathione and NADP+ are produced; the decrease in absorbance at 340 nm is proportional to the enzyme activity (Paglia and Valentine, 1967). Protein content in homogenate islets was measured (Lowry et al., 1951), in order to normalize enzymatic activity. All the spectrophotometric measurements were made in a Jenway 6305 Spectrophotometer (Jenway, Essex, England).

Statistical analysis

Results were analyzed by one-way ANOVA followed by Tukey´s test when was necessary, with SAS 10.0 for Windows. The values were presented as means ± SEM. $P \leq 0.05$ was considered as significant.

Results

Scavenging enzymes immunopresence and TUNEL in rat pancreas

In pancreatic islets from male rats treated with STZ and androgenic derivatives, Cat immunoreactivity was decreased in response to TE, with regard the control group; whereas treatment with DHEA causes an increase in immunopresence; in contrast, treatment with DHT shown the same effect of vehicle alone (Fig. 1).

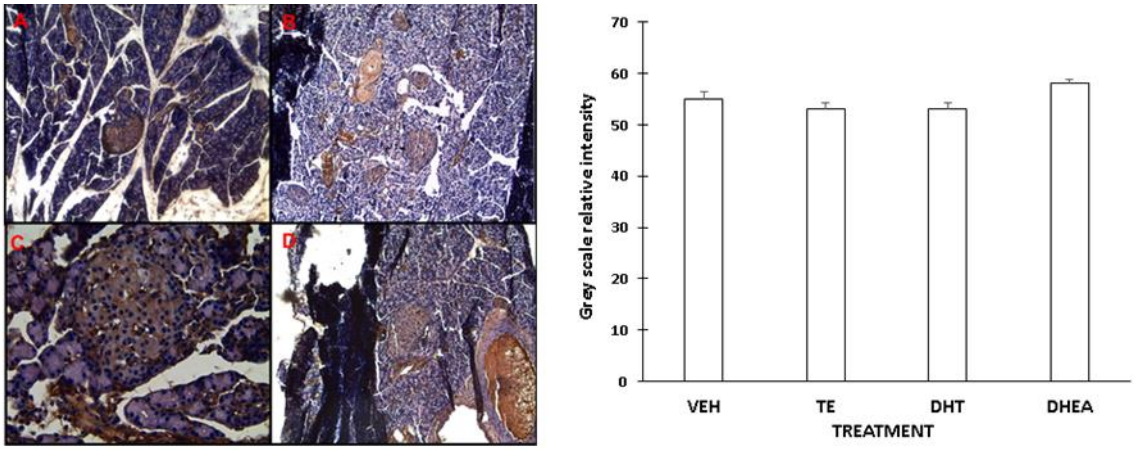

Figure.1. Left: Immunopresence of catalase in islets of male rats treated with STZ, and pre-treated with vehicle (A), testosterone (B), DHT (C), or DHEA (D). Right: Relative intensity (scale of grey) of catalase immunopresence in islets of male rats treated with STZ, and pretreated with steroid hormones. Mean ± S.E.M. of three independent experiments. *P < 0.05 compared with control group.

In the same way, immunopresence of Cu/Zn-SOD was decreased with regard control group after treatment with TE or DHEA, but not with DHT (Fig. 2).

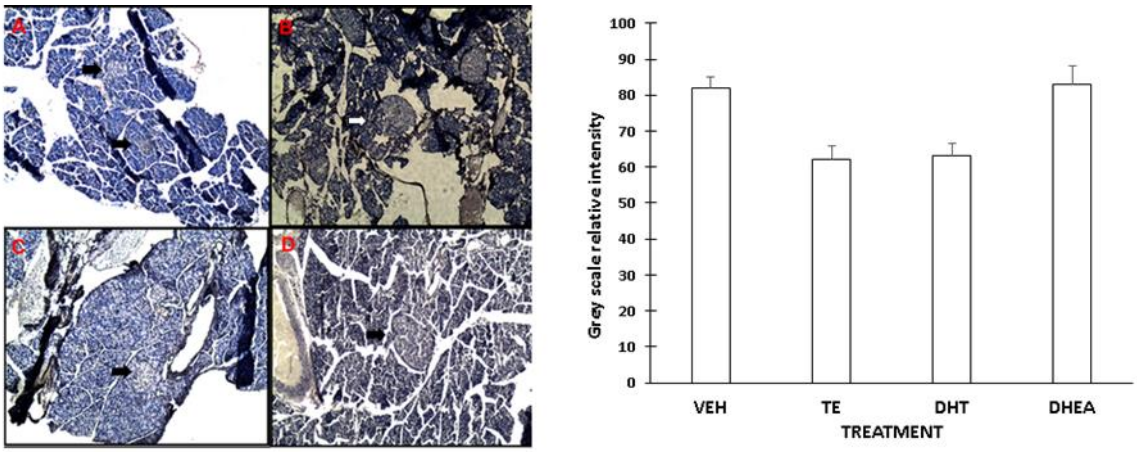

Figure 2. Left: Immunopresence of Cu/Zn-SOD (cytosolic SOD) in islets of male rats treated with STZ, and pre-treated with vehicle (A), testosterone (B), DHT (C), or DHEA (D). Right: Relative intensity (scale of grey) of Cu/Zn SOD immunopresence in islets of male rats treated with STZ, and pretreated with steroid hormones. Mean ± S.E.M. of three independent experiments. *P < 0.05 compared with the control group.

Mn-SOD reactivity of Mn-SOD was non-significant changed, in response to androgen analogs treatment, and highest effect was found with DHEA treatment (Fig. 3).

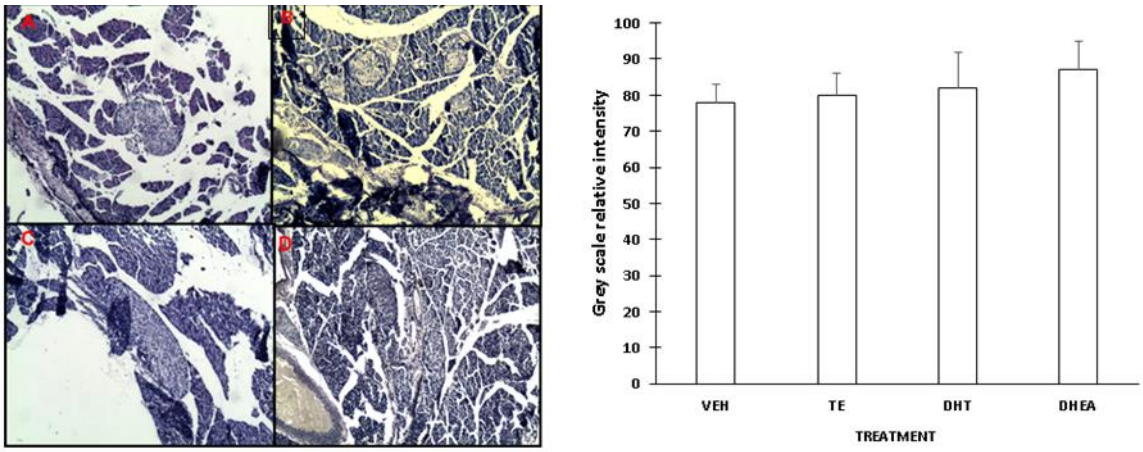

Figure 3. Left: Immunopresence of Mn-SOD (mitochondrial SOD) in islets of male rats treated with STZ, and pre-treated with vehicle (A), testosterone (B), DHT (C), or DHEA (D). Right: Relative intensity (scale of grey) of Mn SOD immunopresence in islets of male rats treated with STZ, and pretreated with steroid hormones. Mean ± S.E.M. of three independent experiments.

TUNEL assay demonstrated that treatment with TE show a notorious decrease with regard the control; apoptosis in vehicle-treated rats was close 30 percent, but in TE and DTH-treated rats, the values are reduced almost 15 percent. DHEA treatment does not decrease apoptosis (Fig. 4).

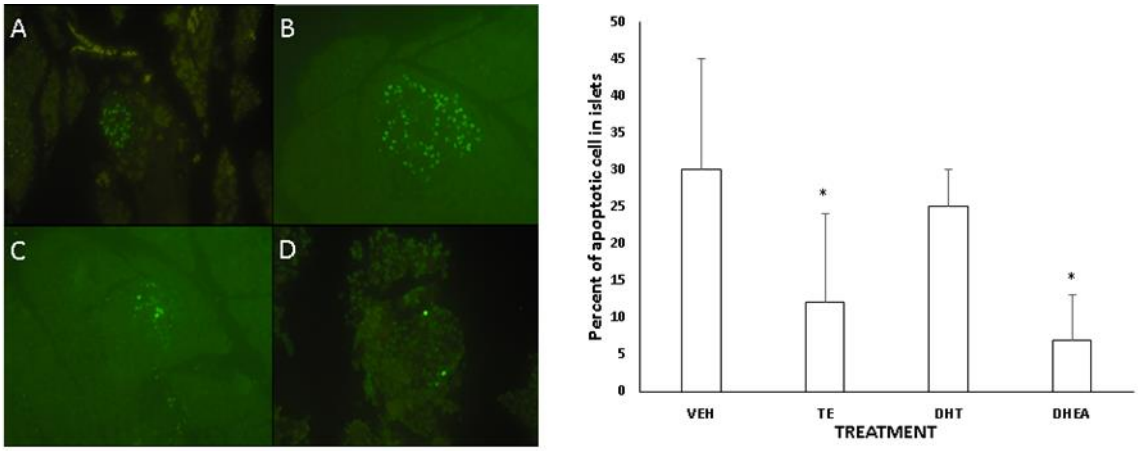

Figure 4. Left: Apoptosis of β-cells in islets of male rats treated with STZ, and pre-treated with vehicle (A), testosterone (B), DHT (C), or DHEA (D). Bright green spots indicate the presence of cells undergo apoptosis, and detected by TUNEL. Right: Percent of apoptosis in islets of male rats treated with STZ, and pretreated with steroid hormones. Mean ± S.E.M. of three independent experiments. *P < 0.05 compared with the control (vehicle) group.

Scavenging enzymes activity in isolated pancreatic islets

The enzyme activity of Cat, SOD (total activity) and GPx were measured in isolated islets from androgen-treated rats free of acinar tissue. Enzymatic activities of scavenging enzymes do not shown any change along the incubation period chosen (6 h). The results are shown in Fig. 5, and although there are variations in enzyme activity, the statistical analysis shown not significant differences, a result that could not be explained by effect of steroid treatment.

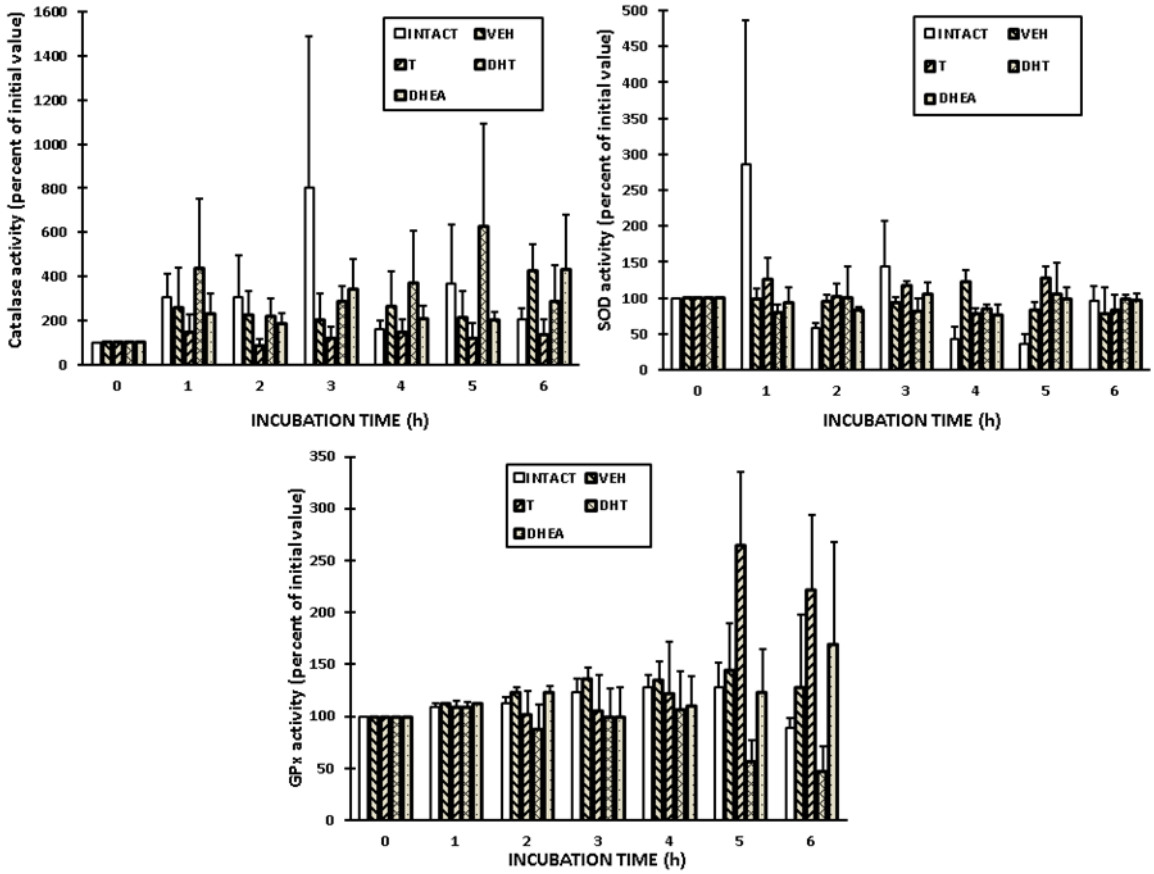

Figure 5. Activity in vitro of antioxidant enzymes in isolated islets of male rats pretreated with steroid hormones, in presence of 6 mM alloxan. The results are expressed as mean ± S.E.M. of three independent experiments. There are not differences in enzyme activity between groups, at any time.

Discussion

Steroid hormones affect cellular susceptibility to oxidative stress (Ahlbom et al., 2001). Thus, testosterone protects cerebellar granular cells from oxidative stress-induced cell death by a recepor-mediated mechanism (Deroo et al., 2004); DHEA exerts a strong antioxidant activity in rats, since it protects various organs against lipid peroxidation (Tam et al., 2003), and prevents the oxidative damage caused by hyperglycemia (Brignardello et al., 1998; Aragno et al., 1999). In rat prostate, testosterone increases the levels of antioxidant enzymes and improves the defensive mechanisms versus oxidative stress (Pang et al., 2002; Tam et al., 2003).

Recently, it has been shown that testosterone protects isolated mouse beta cells to glucose-induced apoptosis. The effect of testosterone suggests amelioration of oxidative stress decreasing in the activity of NADH-oxidase activity (Kooptiwut et al., 2015). Also, testosterone protects INS-1 cells for glucose-induced apoptosis through the reduction of both oxidative and ER stress (Hangchang et al., 2013).

For these reasons, the purpose of this work was to investigate the participation of scavenging enzymes in the protection of pancreatic islets cells after STZ-induced apoptosis, and compare the effect of distinct androgen derivatives in these events. In mice and rats with STZ-induced diabetes, oxidative stress and apoptosis are specific to induce islet destruction (O'Brien et al., 1996; Morimoto et al., 2005). In the rat, testosterone partially prevents the β-cell induced-apoptosis mediated by its androgen receptor (Morimoto et al., 2005). In this work, it was found that DHEA and DHT, besides TE, prevent STZ-induced apoptosis, even higher that the main androgen.

In the other hand, scavenging enzymes as SOD, Cat and GPx, can reduce significantly the levels of ROS in the cells (Poswig et al., 1999). Cu/Zn- and Mn-SOD mRNA increases, but not Cat mRNA, were found in a model of STZ-induced DM2 (Friesen et al, 2004). Our group has been shown that testosterone is a potent cytoprotector of beta-cells in the male but not in the female (Palomar-Morales et al., 2010), and we assume that the ameliorative effect of androgens is mediated by a change in the scavenging enzymes activity; however, since beta-cells are very susceptible to oxidative changes due a low antioxidant capacity and reduced expression of these enzymes (Hotta et al., 1998; Kajimoto and Kaneto, 2004), the experimental approach was to obtain isolated islets, and measure the enzymatic activity in vitro conditions. A time of 6 hours was chosen since in previous reports (Morimoto et al., 2005; Palomar-Morales et al., 2010; Kooptiwut et al., 2015), at this time the highest effect of both STZ and androgens was found. However, it is possible that, in order to show appreciable effects in enzymatic activity of antioxidant enzymes, the time frame should be longer.

Recently, it has been shown that testosterone protects against glucose-induced apoptosis in isolated mouse β-cells. The effect of testosterone appears to be mediated by amelioration of oxidative stress for a decrease in the activity of NADH-oxidase (Kooptiwut et al., 2015). Testosterone protects INS-1 cells for glucose-induced apoptosis, via the reduction of both oxidative stress and ER stress (Hangchang et al., 2013). Also, testosterone increases the insulin secretion of rat pancreatic islets in vitro, and this effect is only seen in male but not in females; additionally 17- β-estradiol or progesterone does not show the same effect (Grillo et al., 2005). These works conduce to further studies to evaluate biochemical and molecular changes inside the islets and/or beta-cells, in order to a better knowledge of diabetes genesis and the possible therapeutic schemes.

Conclusion

In this work, we found that the effects of androgen derivatives over scavenging enzyme immunopresence do not correlate with enzyme activity; the mechanism of cytoprotection of beta-cells by androgens, remains elusive.

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